Vaccine 27 (2009) 4297–4301

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Survival of vaccinated, feed-trained largemouth bass fry (*Micropterus salmoides floridanus*) during natural exposure to *Flavobacterium columnare*

Julie Bebak[a,*,1], Michael Matthews[b,1], Craig Shoemaker[a]

[a] USDA ARS AAHRU, 990 Wire Rd., Auburn, AL 36832, United States
[b] Florida Bass Conservation Center, 3583 CR 788 Webster, FL 33597, United States

ARTICLE INFO

Article history:
Received 23 January 2009
Received in revised form 29 April 2009
Accepted 10 May 2009
Available online 29 May 2009

Keywords:
Largemouth bass
Vaccination
Survival analysis

ABSTRACT

Vaccinated, feed-trained largemouth bass fry (*Micropterus salmoides floridanus*) were cohabited with sham-vaccinated fish. Fish were exposed, under natural conditions, to *Flavobacterium columnare*, a ubiquitous bacterium associated with columnaris disease. During every time interval, the probability that a vaccinated fish would survive past time, *t*, was greater than for sham-vaccinated fish and survivor functions were significantly different (*p*-value <0.001). Overall, vaccinated fish had a 43% lower risk of death during the field trial. Overall incidence was 1.7 times greater for the sham-vaccinated (1.4%/d) as compared to the vaccinated fish (0.8%/d). Vaccination with AQUAVAC-COL (Intervet/Schering-Plough Animal Health) significantly reduced the risk of death from columnaris disease in feed-trained largemouth bass fry.

Published by Elsevier Ltd.

1. Introduction

Flavobacterium spp. are Gram-negative, filamentous, yellow-pigmented bacteria considered to be ubiquitous in the aquatic environment. Worldwide, all fish species are susceptible to infection with at least one member of this genus. One species in particular, *Flavobacterium columnare*, is an important pathogen of freshwater wild, farmed and ornamental fish. Outbreaks of columnaris disease and endemic infection with *F. columnare* have been reported from continents including North America, Europe, and Asia [1–3].

Largemouth bass (*Micropterus salmoides floridanus*; Centrarchidae), a very popular sportfish in the United States, are often reared in government hatchery programs, and then stocked to supplement wild fish populations. After the eggs obtained from broodstock are hatched, fry are stocked into ponds to feed on zooplankton and other small invertebrates. After this period of eating live food (e.g., 30–40 days at 17–24 °C) the fry are more likely to accept commercial feed, so they are harvested from the ponds, stocked into tanks for "feed training", and provided with manufactured feed until they are the size needed for stocking into lakes and ponds. Fry may be exposed to the bacteria while they are in the pond and/or after they are moved indoors, via the water supplied to the tanks. Consequently, outbreaks of columnaris disease are very likely to occur during and after this stressful feed training period. Starvation can increase susceptibility to columnaris disease [4,5], so the fish that do not successfully feed train may be more susceptible to clinical disease. Antibiotic-medicated feed or therapeutants used by immersion can be used to control columnaris outbreaks [6–9], but have limited efficacy, and because of environmental and tissue residue concerns have extremely limited acceptability when used to prevent outbreaks [10].

Prevention of columnaris disease by vaccination is an important goal and a top priority of fish producers throughout the world [11]. Vaccine development for columnaris disease has been in progress for a number of years [e.g., [12–15]]. One result of these efforts has been the development and commercialization of a modified live columnaris immersion vaccine licensed for use in the United States for catfish [16] (AQUAVAC-COL, Intervet/Schering-Plough, *F. columnare* vaccine). This immersion vaccine, which is used at the early fry stage in catfish, might potentially be efficacious for prevention of columnaris disease in largemouth bass fry.

The objective of this study was to conduct a field trial testing the efficacy of AQUAVAC-COL to enhance the survival of vaccinated feed-trained largemouth bass fry naturally exposed to *F. columnare*.

2. Methods

The vaccine trial was conducted at the Florida Bass Conservation Center's Richloam Fish Hatchery (Webster, FL). The water for spawning broodstock and for rearing fry originated as well water, which was collected in a 1.5 acre plastic-lined water reservoir for carbon dioxide degassing and oxygenation before it was supplied

* Corresponding author. Tel.: +1 334 887 3741; fax: +1 334 887 2983.
E-mail address: julie.bebak@ars.usda.gov (J. Bebak).
[1] Both of these authors contributed equally to this work.

0264-410X/$ – see front matter. Published by Elsevier Ltd.
doi:10.1016/j.vaccine.2009.05.026

under flow-through conditions to earthen ponds or to fiberglass culture tanks.

2.1. Pre-trial conditions, vaccination, calcein marking

Largemouth bass fry were obtained from naturally spawning broodstock that produced eggs from January 31 through February 2, 2008. On February 7th (7–9 days post-hatch (dph)), fish were immersion vaccinated with AQUAVAC-COL (Intervet/Schering-Plough) at 1.04×10^8 colony forming units (CFU) *F. columnare*/3.78 L water/20,000 fry for 2 min, then water was added to dilute vaccine to 1.04×10^8 CFU *F. columnare*/18.9 L water/20,000 fry for an additional 30 min. Water temperature was 21.7 °C. On a per vial basis, this exposure was equivalent to using one vial of vaccine to vaccinate 100,000 fry (approximately 340 g total weight) in 18.9 L (5 gallons) of water for 2 min, then adding 75.6 L (20 gallons) of water for an additional 30 min. Sham-vaccinated fry were exposed to modified *Cytophaga* media and glycerol and the same environmental conditions as the vaccinated fry. During vaccination pure oxygen (O_2) was added to maintain dissolved oxygen concentrations ≥6.0 mg/L. After vaccination, 55,000 vaccinated and 55,000 sham-vaccinated fish were stocked into each of two tilled and fertilized 0.24 ha (0.6 acre) earthen ponds.

Fish fed naturally in ponds for 33 d (38–40 dph), until March 11th, were harvested, then stocked into two 9.1 m (30′) long, 4914 L (1300 gallons) fiberglass raceways and trained to accept commercial fish feed (proprietary formulation). In general, fish that successfully accept commercial feed do so within about three days. Fry were then given an additional ten days to recover from handling stress and for those that did not successfully feed train to be culled from the two populations [17,18].

On March 24th (45 d post-vaccination), the sham-vaccinated field trial fish were calcein marked so that sham-vaccinated fish could be distinguished from vaccinated fish during the cohabitation trial [19,20]. Calcein (bis[N,N-bis(carboxymethyl) aminomethyl]fluorescein) (SE-MARK®, Western Chemical, Ferndale, WA), a fluorescent dye with excitation and emission wavelengths of 495 and 515 nm, respectively, chemically binds with alkaline earth metals such as calcium and is considered a safe and effective method for marking fish [20]. Upon binding, calcium-containing structures (e.g., jaw, scales, fin rays) fluoresce green when excited with a blue light (λ = 500 nm). The sham-vaccinated fish were exposed to calcein at a concentration of 2.5 g calcein per liter water for 4 min. Immersion in a 1.5% sodium chloride bath for 4 min prior to calcein immersion was used to increase uptake of the calcein [21] (J. Mohler, personal communication). The vaccinated fish were exposed to the same conditions as the sham-vaccinated fish except that they were not exposed to calcein.

After calcein marking, approximately 1900 sham-vaccinated fish (average body weight ± standard error (SE) = 0.94 ± 0.01 g/fish) were stocked into each of three fiberglass tanks (378.5 L) for the vaccine trial. Vaccinated fish (average body weight ± S.E. = 0.92 ± 0.03 g/fish) were also stocked into each of the same tanks for cohabitation with sham-vaccinated fish, approximately 1900 fish in each tank, for a total of approximately 3800 fish in each tank. Tank assignment for each group stocked was randomly assigned by lottery.

2.2. Vaccine trial conditions

Day 1 of the vaccine trial began two days after acclimation to the experimental tanks. Additional fish that were not feed-trained were culled during this time. Fish were observed for 44 days for signs of mortality due to a natural infection with *F. columnare*. Water quality was measured and recorded daily from the source water and in an individual tank if abnormal fish behavior was observed. Water quality parameters measured (with range of tolerance for largemouth bass) were water temperature (T) = 1–35 °C, dissolved oxygen (DO) = 5–20 mg/L, unionized ammonia (NH_3) = <0.01 mg/L, alkalinity (ALK) = 20–500 mg/L as $CaCO_3$, carbon dioxide (CO_2) = 0–20 mg/L, and pH 6–9 [17,18,22]. Calibrated meters were used to measure T, DO, and pH. Colorimetric methods (Hach Company, Loveland, Colorado) were used to measure NH_3, ALK, and CO_2.

Each day at the same time, dead fish from each tank were examined under a blue light (SE-MARK detector). Employees who were blinded to the treatment assignments counted the fish and assigned them to the appropriate category. Fish were considered dead due to columnaris disease if skin scrapings and microscopic wet mounts were positive or if the erosive or necrotic skin lesions consistent with columnaris disease were apparent [2,23]. Dead fish that were thin with relatively large heads were considered starved (i.e., unsuccessfully feed-trained), and not dead due to columnaris disease. Other causes of death were fish choking while eating tank mates, being partially consumed by a cannibalistic tank mate, and jumping out of the tank. At the end of the trial, the number of fish remaining in each tank was recorded.

2.3. Data analysis

Fish considered dead due to other causes besides columnaris disease were right censored in all analyses. Survivor functions, which estimate the probability that a fish survives beyond time, t, were estimated for each tank. Median survival times, the time beyond which 50% of the fish in an exposure group were expected to survive or the time by which half of the fish had died, were also estimated. The logrank test was used to determine whether survivor functions were significantly different. The logrank test was conducted three times, once for each of the three tanks. Therefore, a Bonferroni correction of $\alpha = 0.05/3 = 0.017$ was used as the cut-off to determine whether the p-value was statistically significant [24,25].

The Cox proportional hazards regression, with clustering by tank, was used to estimate the hazard ratio, or relative risk of dying for sham-vaccinated as compared to vaccinated fish. Before the Cox model was used, the "proportionality assumption" that vaccination multiplies the baseline hazard for the sham-vaccinated fish by a constant factor at any given point during the trial, was tested with an analysis of Schoenfeld residuals. After the Cox regression was estimated, smoothed hazard functions were constructed to examine how the hazard rates varied over time [24–26].

Incidence, the rate at which fish die from columnaris disease, was estimated for exposure groups in each tank. Incidence, with 95% confidence intervals, was calculated as the number of deaths per total fish time at risk. Preventive fraction (PF), with 95% confidence intervals, the net proportion of all deaths in the vaccinated group that were prevented by vaccination, was estimated by $1-(\text{incidence}_{\text{vacc}}/\text{incidence}_{\text{unvacc}})$ [25,27,28].

3. Results

When the 55,000 fish that had been stocked into each of the two ponds on March 11th were harvested, 33,200 unvaccinated, and 29,400 vaccinated fish were recovered. None had gross clinical signs of columnaris disease. During feed training, some sham-vaccinated fish did die with clinical signs of an *F. columnare* infection. However, the emaciated appearance of these fish was compatible with death primarily due to unsuccessful feed training rather than primarily due to columnaris disease. No clinical signs of columnaris disease were observed in vaccinated fish during feed training.

Except for CO_2 and NH_3, water quality parameters remained within normal limits for largemouth bass culture for the entire

44 days. Mean water temperature was 23.4 °C (Standard Error (S.E.) = 0.2), D.O. was 13.9 (S.E. = 0.3) mg/L, alkalinity was 359.8 (S.E. = 2.4) mg/L as $CaCO_3$, and mean pH was 7.58 (S.E. = 0.01). Carbon dioxide was between 8 and 12 mg/L until it increased to 15 mg/L on day 10, after a night time algae bloom die-off in the water reservoir. On the morning of day 12, tank water flow rate was reduced, and the tanks were aerated to dissipate the CO_2. On day 13, NH_3 concentration increased to approximately 1.8 mg/L because of the reduced water flow rate. Normal water flow rates were restored and NH_3 concentrations returned to normal values in less than 1 h. Because it was necessary to maintain water flow rates to avoid NH_3 increases, CO_2 concentrations continued to increase and were between 21 and 29 mg/L until day 31 when they dropped below 20 mg/L after complete decomposition of the dead algae. CO_2 concentrations then remained between 10 and 16 mg/L until the end of the trial. Throughout this period of water quality issues, fish continued to feed and did not exhibit any abnormal behavior.

The number of fish remaining in each tank was counted at the end of the trial. For the purpose of the analysis, at the beginning of the trial tank 1 contained 3620 fish, tank 2 contained 3724 fish, and tank 3 contained 3764 fish, each divided equally between vaccinated and sham-vaccinated groups.

3.1. Survivor functions

For tank 1, the median survival time for the sham-vaccinated fish was 40 days (Fig. 1). For the vaccinated fish in tank 1, the median survival time was not reached by the end of the trial. The sham-vaccinated fish had a 0.47 probability of surviving beyond the end of the trial. The vaccinated fish had a 0.68 probability of surviving beyond the end of the trial. The logrank test indicated that the two survivor curves were significantly different from each other (χ^2, 1 d.f. = 148.65; p-value < 0.0001).

For tank 2, the median survival time for the sham-vaccinated fish was 26 days. By the end of the trial, vaccinated fish had not reached the median survival time, i.e., greater than 50% of the fish still remained. The sham-vaccinated fish had a 0.39 probability of surviving beyond the end of the trial. The vaccinated fish had a 0.56 probability of surviving beyond the end of the trial. The two survivor curves were significantly different from each other (χ^2, 1 d.f. = 89.51; p-value < 0.0001).

Overall, survival in tank 3 was greater than in tank 1 or tank 2. Neither the sham-vaccinated or vaccinated fish reached the median survival time by the end of the trial. However, the sham-vaccinated fish reached the time by which 25% (1st quartile) of the fish would have died, 28 days, while the vaccinated fish did not reach that quartile before the end of the trial. The sham-vaccinated fish had a 0.64 probability of surviving beyond the 44 day trial period. Vaccinated fish had a 0.78 probability of surviving beyond 44 days. The two survivor curves were significantly different from each other (χ^2, 1 d.f. = 101.20; p-value < 0.0001).

During every time interval for every tank, the probability that a vaccinated fish would survive past time, t, was always greater than it was for a sham-vaccinated fish (Fig. 1).

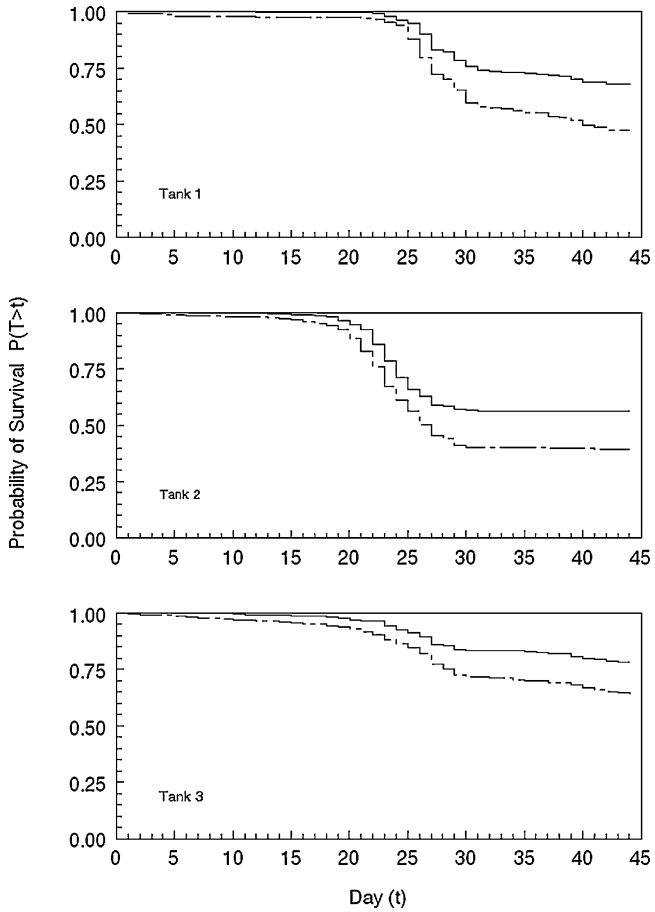

Figure 1. Probability of survival past time, t (P ($T > t$)), for vaccinated or sham-vaccinated, feed-trained, largemouth bass fry (– – – sham-vaccinated; — vaccinated).

3.2. Cox proportional hazards model

Before estimating the Cox proportional hazards model, the proportional hazards assumption was tested by examining Schoenfeld residuals. This test was not statistically significant ($\chi^2 = 0.27$, 1 d.f., p-value = 0.60), so the Cox proportional hazards model was used. The hazard ratio for vaccination compared with no vaccination was 0.57 (Robust S.E. = 0.03; 95% C.I. = 0.51, 0.64). Vaccination had a statistically significant protective effect. Vaccinated fish had 57/100

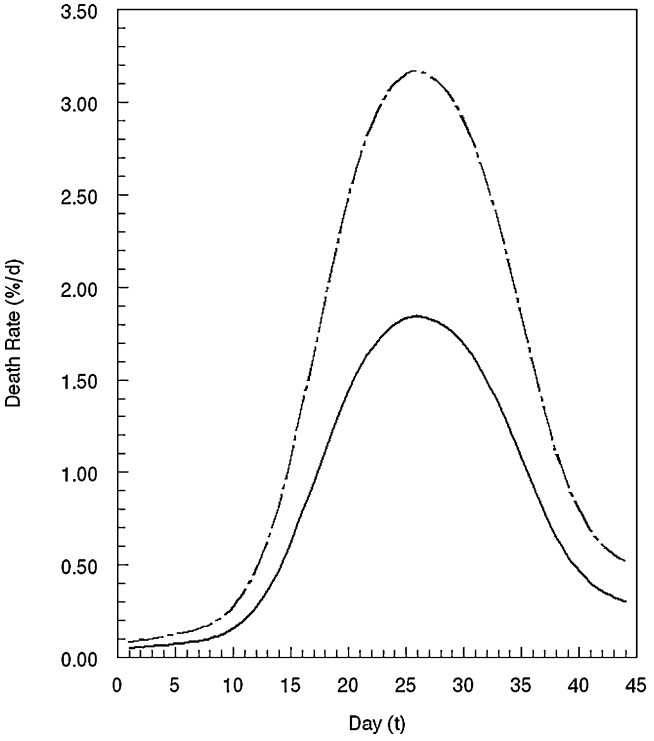

Figure 2. Smoothed hazard functions comparing death rate (%/d) for vaccinated or sham-vaccinated feed-trained largemouth bass fry (– – – sham-vaccinated; — vaccinated).

Table 1
Incidence and overall incidence plus lower and upper boundaries for 95% confidence intervals (Lower 95% C.I., Upper 95% C.I.) for each of the field trial groups in each of the tanks containing feed-trained largemouth bass fry.

	Sham-vaccinated		Vaccinated	
	Incidence	95% CI (lower, upper)	Incidence	95% CI (lower, upper)
Tank 1	0.0147	0.0138, 0.0157	0.0081	0.0074, 0.0088
Tank 2	0.0191	0.0179, 0.0202	0.0119	0.0111, 0.0128
Tank 3	0.0096	0.0089, 0.0104	0.0053	0.0048, 0.0058
Overall	0.0142	0.0136, 0.0147	0.0082	0.0079, 0.0087

Sham-vaccinated and vaccinated fish were cohabited in each tank.

the hazard of the sham-vaccinated fish. In other words, when compared to the sham-vaccinated fish, vaccinated fish had a 43% lower risk of death during the trial.

Smoothed hazard functions obtained from the Cox proportional hazards regression indicated that the instantaneous measure of death rate increased, then peaked at 3.2%/d and at 1.8%/d on day 26 for sham-vaccinated and vaccinated fish, respectively, then declined (Fig. 2). At every point in time, the death rate was lower for vaccinated than for sham-vaccinated fish. At 44 days, the death rate was 0.6%/d for sham-vaccinated fish and 0.4%/d for vaccinated fish.

3.3. Incidence and preventive fraction

Incidence, the rate at which fish died from columnaris disease was greater, for all tanks, for sham-vaccinated as compared to vaccinated fish (Table 1). Overall incidence was 1.7 times greater for the sham-vaccinated (1.4%/d) as compared to the vaccinated fish (0.8%/d). Confidence intervals (95%) for incidence were non-overlapping for comparison of sham-vaccinated and vaccinated fish for each tank and for the overall comparison.

The point estimates for preventive fraction, net proportion of all deaths in the vaccinated fish that were prevented by vaccination, were 0.45 (95% C.I. = 0.39, 0.51), 0.37 (95% C.I. = 0.31, 0.43), and 0.45 (95% C.I. = 0.37, 0.52) for tanks 1–3, respectively.

4. Discussion

In this field trial, AQUAVAC COL provided feed-trained largemouth bass fry with significant protection from mortality during natural challenge with *F. columnare*. Throughout the trial, in all three tanks containing cohabited sham-vaccinated and vaccinated fish, the risk of death was always higher for sham-vaccinated than for vaccinated fish. Statistical testing of survivor functions, Cox regression analysis, and estimates of incidence and preventive fraction for each tank demonstrated that differences were significant.

The vaccine afforded significant protection even though fry were vaccinated at just 7–9 dph. Fish, in general, possess a non-adaptive immune system at the eyed egg stage (24–48 h prior to and at hatch) [29,30,15], and in the 4-day-old yolk sac larvae (about 4–7 days after hatching) [31,29]. Immunoglobulin, lectins, lysozyme, C-reactive protein and other antimicrobial substances are present in the fish egg and fry [32–36]. Macrophages are reported to be present in the skin and intestines of four-day-old fry [31]. Recently, Peterson et al. [37] suggested that innate recognition genes (i.e., toll-like receptors) are present during embryogenesis in catfish. These non-adaptive defense mechanisms are important for survival of eggs and fry in hostile environments of pathogenic and opportunistic microorganisms. Ingestion of microorganisms by fish fry or attachment of microorganisms to the surface of eggs may be important for priming the early immune system [38].

Antigen priming and adaptive recognition of a specific pathogen is likely the result of the presence of specific microorganisms in or on the eggs and fry of fish. Rombout et al. [39] provide evidence for appearance of the first plasma cell in cyprinids and marine fish. This immune cell first appears after the first intake of food in cyprinids and around the first intake of food in marine fish. The first appearance of IgM on lymphocytes is later in marine species than in fresh water species [40]. In channel catfish, Petrie-Hanson and Ainsworth [29] demonstrated IgM positive lymphocytes on day 7 post-hatch in the renal hematopoietic tissue. The adaptive immune system develops shortly thereafter and matures as the fish increase in size and age. Maturity of the adaptive immune system is generally believed to be at or around 28 days depending on species of fish and water temperature [41]. Protection induced by vaccination of immunologically immature animals is believed to result due to the persistence of the modified live vaccine (i.e., until the immune system can respond) and/or the modified live vaccine results in expression of antigens with the correct MHC on host immune cells, thus activating immunocompetent cells [42].

During the field trial CO_2 and NH_3 concentrations increased above normal limits for about 20 days and for less than one day, respectively. If a dead fish had no clinical signs of columnaris disease or a negative microscopic exam, then it would have been censored in the analysis if it simply died from NH_3 or CO_2 toxicity. For fish infected with *F. columnare*, these stressors may have increased the death rate somewhat. However, death rate always remained lower for the vaccinated fish. In largemouth bass culture, increases in CO_2 and NH_3 are common occurrences because most producers use pond water reservoirs as part of the water supply. These results provide largemouth bass producers with the assurance that, in a well-managed system, vaccination will provide protection even when fish are subjected to common stressors during the production cycle.

Acknowledgements

The authors gratefully acknowledge Mr. Josh Sakmar and Mr. Justin Elkins for excellent technical assistance. Intervet/Schering-Plough Animal Health supplied the vaccine for this study. This work was partially supported by the US Department of Agriculture, Agricultural Research Service, Current Research Information Systems Project No. 6420-32000-022-00D. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

References

[1] Wakabayashi H. Columnaris disease. In: Inglis V, Roberts RJ, Bromage NR, editors. Bacterial diseases of fish. Oxford, England: Blackwell Science Ltd.; 1993. p. 23–39.
[2] Shotts EB, Starliper CE. Flavobacterial diseases: columnaris disease, cold-water disease and bacterial gill disease. In: Woo PTK, Bruno DW, editors. Fish diseases and disorders, volume 3: viral, bacterial and fungal infections. Oxon: CABI Publishing; 1999. p. 559–76.
[3] Olivares-Fuster O, Baker JL, Terhune JS, Shoemaker CA, Klesius PH, Arias CR. Host-specific association between *Flavobacterium columnare* genomovars and fish species. Systemic and Applied Microbiology 2007;30:624–33.
[4] Klesius P, Lim C, Shoemaker C. Effect of feed deprivation on innate resistance and antibody response to *Flavobacterium columnare* in channel catfish, Ictalurus punctatus. Bulletin of the European Association of Fish Pathologists 1999;19:156–8.
[5] Shoemaker CA, Klesius PH, Lim C, Yildirim M. Feed deprivation of channel catfish, *Ictalurus punctatus* (Rafinesque), influences organosomatic indices, chemical composition and susceptibility to *Flavobacterium columnare*. Journal of Fish Diseases 2003;26:553–61.
[6] Steeger TM, Grizzle JM, Weathers K, Newman M. Bacterial diseases and mortality of angler-caught largemouth bass released after tournaments on Walter F. George Reservoir, Alabama-Georgia. North American Journal of Fisheries Management 1994;14:435–41.
[7] Gaikowski MP, Rach JJ, Ramsay RT. Acute toxicity of hydrogen peroxide treatments to selected lifestages of cold-, cool-, and warmwater fish. Aquaculture 1999;178:191–207.
[8] Tidwell JH, Coyle SD, Woods TA. Species profile: largemouth bass. Stoneville, MS: Southern Regional Aquaculture Center; 2000 (SRAC Publication No. 722).

[9] Gaikowski MP, Larson WJ, Gingerich WH. Survival of cool and warm freshwater fish following chloramine-T exposure. Aquaculture 2008;275:20–5.
[10] Chinabut S, Puttinaowarat S. The choice of disease control strategies to secure international market access for aquaculture products. Developmental Biology (Basel) 2005;121:255–61.
[11] Shoemaker CA, Klesius PH, Evans JJ. Modified live *Flavobacterium columnare* against columnaris disease in fish. United States Patent No. 6,881,412 B1; 2005.
[12] Thune RL, Collins LA, Pena MP. A comparison of immersion, immersion/oral combination and injection methods for the vaccination of channel catfish, *Ictalurus punctatus*, against *Edwardsiella ictaluri*. Journal of the World Aquaculture Society 1997;28:193–201.
[13] Hu C, Hong Y, Lin G. Studies on the variation of immune cells in Xingguo red carp, *Cyprinus carpio* var. singuonensis, immunized by *Cytophaga columnaris*. Acta hydrobiologica sinica 2002;26:674–8.
[14] Grabowski LD, LaPatra SE, Cain KD. Systemic mucosal antibody response in tilapia, *Oreochromis niloticus* (L.), following immunization with *Flavobacterium columnare*. Journal of Fish Diseases 2004;27:573–81.
[15] Shoemaker CA, Klesius PH, Evans JJ. Immunization of eyed channel catfish, *Ictalurus punctatus*, eggs with monovalent *Flavobacterium columnare* vaccine and bivalent *F. columnare* and *Edwardsiella ictaluri* vaccine. Vaccine 2007;25:1126–31.
[16] Shoemaker CA, Klesius PH, Evans JJ, Arias CR. Use of modified live vaccines in aquaculture. Journal of the World Aquaculture Society; in press.
[17] Stickney RR. Culture of nonsalmonid freshwater fishes. Boca Raton, FL: CRC Press; 1986.
[18] U.S. Fish and Wildlife Service. Third Report to Fish Farmers. Washington, DC: US Department of the Interior; 1984.
[19] Honeyfield DC, Ostrowski CS, Fletcher JW, Mohler JW. Dietary calcein marking of brook trout, Atlantic salmon, yellow perch, and coho salmon scales. North American Journal of Fisheries Management 2006;26:431–7.
[20] Klesius PH, Evans JJ, Shoemaker CA, Pasnik DJ. A vaccination and challenge model using calcein marked fish. Fish and Shellfish Immunology 2006;20:20–8.
[21] Mohler J. Producing fluorescent marks on Atlantic salmon fin rays and scales with calcein via osmotic induction. North American Journal of Fisheries Management 2003;23:1108–13.
[22] Boyd CE. Water quality in ponds for aquaculture. Auburn: Alabama Experimental Station; 1990.
[23] Noga EJ. Bacterial diseases of temperate freshwater and estuarine fishes. In: Stoskopf MK, editor. Fish medicine. Philadelphia: WB Saunders Co.; 1993. p. 269–78.
[24] Kleinbaum DG. Survival analysis. A self-learning text. New York: Springer-Verlag Inc.; 1996.
[25] StataCorp. Stata Statistical Software: Release 9. College Station, TX: StataCorp. LP; 2005.
[26] Cleves MA, Gould WW, Gutierrez RG. An Introduction to survival analysis using stata. Revised Edition College Station, Texas: Stata Press; 2004.
[27] Kleinbaum DG, Kupper LL, Morgenstern H. Epidemiologic research. New York: Van Nostrand Reinhold; 1982.
[28] Rothman KJ. Modern epidemiology. Boston: Little, Brown; 1986.
[29] Petrie-Hanson L, Ainsworth AJ. Ontogeny of channel catfish lymphoid organs. Veterinary Immunology and Immunopathology 2001;81:113–27.
[30] Shoemaker CA, Klesius PH, Evans JJ. *In ovo* methods for utilizing the modified live *Edwardsiella ictaluri* vaccine against enteric septicemia in channel catfish. Aquaculture 2002;203:221–7.
[31] Grace MF, Botham JW, Manning MJ. Ontogeny of lymphoid organ function in fish. In: Aspects of developmental and comparative immunology: proceedings of the 1st congress of developmental and comparative immunology. Oxford: Pergamon Press; 1981. p. 467–8.
[32] Nosek J, Krajhanzl A, Kocourek J. Studies on lectins. LVII. Immunofluorescence localization of lections present in fish ovaries. Histochemistry 1983;79:131–9.
[33] Kanlis G, Suzuki Y, Tauchi M, Numata T, Shirojo Y, Takashima F. Immunoglobulin concentration and specific antibody activity in oocytes and eggs of immunized red sea bream. Fisheries Science 1995;61:791–5.
[34] Kanlis G, Suzuki Y, Tauchi M, Numata T, Shirojo Y, Takashima F. Immunoglobulin in oocytes, fertilized eggs, and yolk sac larvae of red sea bream. Fisheries Science 1995;61:787–90.
[35] Yousif AN, Albright LJ, Evelyn TPT. Occurrence of lysozyme in the eggs of coho salmon *Oncorhynchus kisutch*. Diseases of Aquatic Organisms 1991;10:45–9.
[36] Yousif AN, Albright LJ, Evelyn TPT. Purification and characterization of a galactose-specific lectin from the eggs of coho salmon *Oncorhynchus kisutch* and its interaction with bacterial fish pathogens. Diseases of Aquatic Organisms 1995;20:127–36.
[37] Peterson BC, Bosworth BG, Bilodeau AL. Differential gene expression of IGF-I, IGF-II, and toll-like receptors 3 and 5 during embryogenesis in hybrid (channel × blue) and channel catfish. Comparative Biochemistry and Physiology Part A Molecular Integrative Physiology 2005;141:42–7.
[38] Rombout JHWM, Berg AAvd. Immunological importance of the second gut segment of carp. I. Uptake and processing of antigens by epithelial cells and macrophages. Journal of Fish Biology 1989;35:13–22.
[39] Rombout JHWM, Huttenhuis HBT, Picchietti S, Scapigliati G. Phylogeny and ontogeny of fish leucocytes. Fish Shellfish Immunology 2005;19:441–55.
[40] Magnadottir B, Lange S, Gudmundsdottir S, Bøgwald J, Dalmo RA. Ontogeny of humoral immune parameters in fish. Fish Shellfish Immunology 2005;19:429–39.
[41] Ellis AE. Ontogeny of the immune system in teleost fish. In: Ellis AE, editor. Fish vaccination. London: Academic Press; 2001. p. 20–31.
[42] Mast J, Goddeeris BM. Development of immunocompetence of broiler chickens. Veterinary Immunology and Immunopathology 1999;70:245–56.